Characterization of Effector Cells Mediating Antitumor Activity in Spleen Cells of Tumor-bearing Mice

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ABSTRACT

Antitumor activity of spleen cells from BALB/c mice bearing RL31 lymphoma was studied. In the Winn assay spleen cells of the tumor-bearing mice inhibited the growth of RL31 lymphoma. This antitumor activity of spleen cells was not detected by the in vitro cell-mediated cytotoxicity assay (4-h 51Cr release assay). The effector cells in spleen cells were T-cells which manifested asialo GM1 on their cell surfaces and were radiosensitive (1000 rads). The analysis of T-cell subsets using Lyt markers showed that the effector cells expressed the Lyt-2 antigen with a small amount of the Lyt-1 antigen on their cell surfaces. In addition antitumor activity of spleen cells of the tumor-bearing mice was weak in the early stage of tumor growth, strong in the mid-stage, and disappeared in the late stage. The mechanisms of antitumor activity of spleen cells of the tumor-bearing mice are discussed.

INTRODUCTION

A variety of cell populations has been demonstrated to be involved in immunological responses against tumor. Among various cell populations, T-cells appear to play the central role in the antitumor responses (1). It has been generally accepted that CTLs1 may be the main effector cells in graft or tumor rejection (2). However, studies denying the role of CTLs in graft or tumor rejection have been recently presented (3). In mice, T-cells can be phenotypically classified by their expression of Lyt differentiation antigens. The analysis of T-cell subsets using these markers indicates in several systems that the Lyt-1-2-2+ CTLs are not involved in graft or tumor rejection but the Lyt-1-2-2+ T-cell subset is essential for these immune responses (4–7). Although we cannot ignore these reports the actual lymphocyte subpopulations involved in the cell-mediated immune responses against graft or tumor remain to be identified.

In this work to circumvent these problems we investigated which mechanisms are responsible for the in vivo immune resistance against tumor in the tumor-bearing mice. The experiments reported herein characterized the nature and surface phenotype of the effector cells exhibiting antitumor activity in spleen cells of the tumor-bearing mice. The antitumor activity of the effector cells was detected by the in vivo tumor-neutralization assay (Winn assay). The effector cells were T-cells which expressed asialo GM1 on their cell surfaces and were sensitive to irradiation of 1000 rads. The analysis of T-cell subsets demonstrated that the effector cells manifested the Lyt-2 antigen with a small amount of the Lyt-1 antigen on their cell surfaces. The results indicate that the T-cell subset of the CTLs or CTL-lineage cells is involved in the immune resistance against tumor in the tumor-bearing mice. Moreover the data suggest that in spleens of the tumor-bearing mice the maturation of the CTLs may be blocked.

MATERIALS AND METHODS

Mice. BALB/c mice 7 to 10 weeks old were obtained from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Hamamatsu, Shizuoka-ken, Japan). In all experiments the mice were sex- and age-matched and the results obtained with both sexes were identical.

Tumors. RL31 is a radiation-induced lymphoma of BALB/c mice and was maintained in vivo as ascitic cells. YAC-1 is a Moloney virus-induced lymphoma of A/Sn mice and was maintained by continuous in vitro culture in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). Meth A is a methylcholanthrene-induced fibrosarcoma of BALB/c mice and was maintained in vivo as ascitic cells.

Preparation of Spleen Cells. Single cell suspensions were prepared from spleens of the normal or tumor-bearing mice by glass homogenizers and washed three times with MEM. Separation of spleen cells over nylon wool columns was performed as described by Julius et al. (8).

Winn Assay. Spleen cells were suspended in MEM and adjusted to a concentration of 5 or 10 x 10⁶ viable cells/ml. The spleen cells were mixed with tumor cells (1 or 2 x 10⁶/ml) suspended in MEM at a 100:1 spleen cell-to-tumor cell ratio. This mixture in a total volume of 0.15 ml was injected s.c. into the shaved backs of mice. Each group of mice consisted of 6 mice. For each experiment controls were constituted by the mixture of normal spleen cells and tumor cells or by tumor cells alone. After inoculation mice were inspected every 3 days for tumor appearance and growth. The growth of tumor was determined by measuring two bisecting diameters of each tumor.

Cell-mediated Cytotoxicity Assay. Chromium-release cytotoxicity assays were performed in microplates as described previously (9). In brief 51Cr-labeled target cells (2 x 10⁴) were added to the effector cells in a total volume of 0.2 ml. After a 4-h incubation the plates were centrifuged and the radioactivity of 0.1 ml of the supernatant was measured in a gamma counter. As baseline controls the same numbers of unlabeled target cells instead of effector cells were added to the labeled target cells. The maximum release was determined by adding 1 N HCl to the labeled target cells. Percentage of lysis was calculated by means of the following formula:

Lysis (%) = \[ \frac{\text{Release from experimental group} - \text{release from baseline control}}{\text{Maximum release} - \text{release from baseline control}} \times 100 \]

All experimental points were shown by the mean ± SE of triplicate samples.

Treatment of Spleen Cells with Rabbit Anti-Asialo GM1 or Monoclonal Anti-Thy-1.2 Antiserum and Complement. Spleen cells (3 x 10⁶) were incubated for 30 min at 4°C with 1 ml of rabbit anti-asialo GM1 antiserum (a gift of Dr. K. Okumura, Department of Immunology, Tokyo University School of Medicine) at a final dilution of 1:40 or 1 ml of...
monoclonal anti-Thy-1.2 antibody (Olcac, Ltd., Bicester Oxon, England) at a final dilution of 1:400. The cells were then pelleted and the supernatant was discarded and 1 ml of a 1:4 dilution of guinea pig complement was added. After 30 min at 37°C the cells were washed three times with MEM.

Treatments of Spleen Cells with Monoclonal Anti-Lyt-1.2 or Anti-Lyt-2.2 Antibody and Complement. Spleen cells (1 x 10^6) were treated for 60 min at 4°C with 1 ml of monoclonal anti-Lyt-1.2 or anti-Lyt-2.2 antibody (Cedarlane Laboratories, Ltd., Hornby, Ontario, Canada) at a final dilution of 1:10 or 1:400, respectively. After that, the cells were pelleted and resuspended in 1 ml of a 1:10 dilution of rabbit complement (Cedarlane Laboratories) for 60 min at 37°C and then washed three times with MEM.

In Vitro Induction of Cytotoxic T-Lymphocytes against RLd1. The generation of cytotoxic T-lymphocytes specific for RLd1 was performed by a modification of the method of Nakayama et al. (10). Briefly responder spleen cells were obtained from BALB/c mice inoculated s.c. with 5 x 10^6 RLd1 cells 14 days previously. Responder spleen cells (4 x 10^6) were cultured with 4 x 10^6 stimulator RLd1 cells (irradiated with 10,000 rads) in the Nunclon culture plates (multidish 6-well plates) in a total volume of 8 ml. The culture medium was RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 5 x 10^-4 M 2-mercaptoethanol, penicillin (100 units/ml), streptomycin (100 mM/ml) and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. Cultures were incubated at a final dilution of 1:400. The cells were then pelleted and resuspended in 1 ml of a 1:10 dilution of rabbit complement (Cedarlane Laboratories) for 60 min at 37°C and then washed three times with MEM.

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Results

Antitumor Activity of Spleen Cells of RLd1-bearing Mice. After BALB/c mice were inoculated s.c. with 5 x 10^6 RLd1 cells all of these mice had progressive tumor growth and died within a period of 30 days. When spleen cells from the tumor-bearing mice which were inoculated s.c. with 5 x 10^6 RLd1 cells 14 days previously were admixed with RLd1 cells and the mixtures were inoculated s.c. into normal BALB/c mice, we observed complete inhibition of tumor growth (Chart 1A). However, spleen cells from the normal or Meth A-bearing mice did not inhibit the growth of RLd1 (Chart 1A). In addition spleen cells of the RLd1-bearing mice were not able to suppress the growth of Meth A, which was a syngeneic tumor of BALB/c mice (Chart 1B).

The in vitro cytotoxic activity of the spleen cells used in the experiment of Chart 1 was tested in a 4-h 51Cr release assay (Table 1). YAC-1 cells were used as the target cells for NK activity. The spleen cells from the Meth A-bearing mice showed the highest levels of cytotoxicity against RLd1 and YAC-1 cells in three populations of the effector cells. The cytotoxic activities of spleen cells from the normal and RLd1-bearing mice, respectively, were almost equal. There was no correlation between the in vivo protective activity and the in vitro cytotoxic activity of spleen cells.

Surface Phenotypes of Effector Cells Displaying Antitumor Activity. The whole spleen cell population of the tumor-bearing mice protected the recipient mice from tumor growth (Chart 1A) and the nylon wool-passed population of spleen cells similarly gave protection (data not shown). This suggested that neither macrophages nor B-cells in spleen cells of the tumor-bearing mice provided protection against tumor growth.

To further characterize the effector cells spleen cells of the tumor-bearing mice were treated with monoclonal anti-Thy-1 or rabbit anti-asialo GM1 antiserum and complement before s.c. transfer with tumor cells (Chart 2A). Treatment of spleen cells of the tumor-bearing mice with anti-Thy-1 or anti-asialo GM1 serum and complement destroyed largely the protective effect of spleen cells. Complement alone had no effect on the protective activity of spleen cells. This result indicates that the protective ability of spleen cells of the tumor-bearing mice is dependent on Thy-1 and asialo GM1 positive cells.

Next spleen cells from the tumor-bearing mice were treated with monoclonal antibodies against Lyt surface antigens before testing antitumor activity of spleen cells in the Winn assay (Chart 2B). Treatment of spleen cells from the tumor-bearing mice with monoclonal anti-Lyt-2 antisera and complement almost completely abrogated their ability to inhibit the tumor growth. On the other hand mice that received the spleen cells treated with anti-Lyt-1 antisera and complement did not have the appearance of tumor until day 18; however, 2 of 6 mice developed tumors 21 days later (Chart 2B). Thus the effector cells mediating antitumor activity had at least the Lyt-2 antigen on their cell surfaces.

Surface Phenotypes of Cytotoxic Effector Cells in Vitro Generated against RLd1. As described in "Materials and Methods" Nakayama et al. (10) reported that the CTLs specific for RLd1 cells were induced from in vivo primed and in vitro restimulated spleen cells. As shown in Table 2 the cytotoxic activity of the cytotoxic effector cells induced by the secondary MLTC was almost completely abolished by treatment with monoclonal anti-Thy-1 or anti-Lyt-2 antisera and complement. In addition treatment of the effector cells with monoclonal anti-Lyt-1 antiserum and complement greatly reduced their cytotoxic activity but a little of cytotoxic activity remained (Table 2, Experiment 2). On the other hand the effector cells treated with rabbit anti-asialo GM1 serum and complement only slightly reduced their cytotoxic activity (Table 2, Experiment 1). These results indicate that the CTLs induced by the secondary MLTC are Lyt-1^+^2^+^ T-cells and express very low levels of asialo GM1 antigen on their cell surfaces.

Radiation Sensitivity of Effector Cells Responsible for Antitumor Activity. When spleen cells of the tumor-bearing mice were irradiated with 1000 rads before s.c. transfer, the antitumor activity of spleen cells was eliminated completely (Chart 3). This result suggests that proliferation and DNA synthesis of the effector cells are required for the manifestation of their antitumor activity. On the other hand mice that received the spleen cells of the tumor-bearing mice completely inhibited the tumor growth in the Winn assay (Chart 4).

Simultaneously the in vitro cytotoxic activities of these spleen cells against RLd1 and YAC-1 cells were tested in a 4-h 51Cr release assay (Table 3). The spleen cells from the 14-day tumor-bearing mice completely inhibited the tumor growth in the Winn assay. However, spleen cells on day 6 of tumor growth showed a weakly protective effect and were not able to inhibit the tumor growth.
ANTITUMOR ACTIVITY OF SPLEEN CELLS OF TUMOR-BEARING MICE

30
20
10
0

Mean tumor diameter (mm)

30
20
10
0

Mean tumor diameter (mm)

0 6 12 18 24 30
Days after inoculation

0 6 12 18 24 30
Days after inoculation

A

B

Table 1
Cytotoxic activity of spleen cells from normal, RL61-, and Meth A-bearing mice against RL61 and YAC-1 cells

<table>
<thead>
<tr>
<th>Source of effector cells</th>
<th>Effector:target cell ratio</th>
<th>Percent lysis of target cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>RL61</td>
</tr>
<tr>
<td>Normal BALB/c mice</td>
<td>100:1</td>
<td>3.3 ± 0.7*</td>
</tr>
<tr>
<td></td>
<td>50:1</td>
<td>4.1 ± 0.8</td>
</tr>
<tr>
<td>RL61-bearing BALB/c</td>
<td>100:1</td>
<td>5.7 ± 1.1</td>
</tr>
<tr>
<td>mice</td>
<td>50:1</td>
<td>4.4 ± 1.1</td>
</tr>
<tr>
<td>Meth A-bearing BALB/c</td>
<td>100:1</td>
<td>8.3 ± 1.9</td>
</tr>
<tr>
<td>mice</td>
<td>50:1</td>
<td>5.4 ± 0.3</td>
</tr>
</tbody>
</table>

* Mean ± SE of triplicate samples.

growth completely. The inhibitory activity of spleen cells from the tumor-bearing mice disappeared on day 22 of tumor growth (Chart 4). On the other hand the in vitro cytotoxic activity of spleen cells against RL61 cells on day 14 of tumor growth was not different from that on days 6 and 22 of tumor growth and that of normal mice (Table 3). In addition NK activity of spleen cells (cytotoxic activity against YAC-1 cells) was slightly enhanced in the 6-day tumor-bearing mice but almost completely suppressed in the 22-day tumor-bearing mice.

DISCUSSION

This study showed that tumor-bearing mice had in their spleen cells effector T-cells displaying antitumor activity against the same tumor. As shown in Chart 4 the antitumor activity of spleen cells from the tumor-bearing mice was weak in the early stage of tumor growth (day 6 of tumor growth), strong in the midstage (day 14 of tumor growth), and disappeared in the late stage (day 22 of tumor growth). There was no correlation between the in vivo antitumor activity tested in the Winn assay and the in vitro cell-mediated cytotoxic activity examined by a 4-h 51Cr release assay (Charts 1 and 4; Tables 1 and 3). We attempted to determine which T-cell subclass defined by cytolysis with monoclonal anti-Lyt-1 or anti-Lyt-2 antisera and complement is required to transfer adoptively the ability to inhibit the growth of RL61 lymphoma (Chart 2B). Protective activity of spleen cells from the tumor-bearing BALB/c mice against the growth of RL61 lymphoma was shown to be mediated by T-cells expressing at least the Lyt-2 antigen on their cell surfaces (Chart 2B). Some of them seemed to display the Lyt-1 antigen because treatment of spleen cells with anti-Lyt-1 antiserum and complement partially reduced their antitumor activity. Recent studies show that most T-cells express the Lyt-1 antigen to some degree (11). Our
ANTITUMOR ACTIVITY OF SPLEEN CELLS OF TUMOR-BEARING MICE

Chart 2. Effect of pretreatment with antibodies to lymphoid-cell surface markers and complement on antitumor activity of spleen cells from RL61-bearing mice in the Winn assay. A, spleen cells from RL61-bearing mice (day 14 of tumor growth) untreated (a) or pretreated with anti-Thy-1.2 and complement (D), anti-asialo GM1 and complement (C), and complement alone (C) before the Winn assay. B, spleen cells from RL61-bearing mice pretreated with anti-Lyt-1.2 and complement (D), anti-Lyt-2.2 and complement (C), and complement alone (A). Control mice received RL61 cells (1 x 10⁶) alone (a) and the mixture of RL61 cells (5 x 10⁶) and spleen cells from normal mice (C). Each group of recipients consisted of 6 mice. The spleen:tumor cell ratio was 100:1. Bars, SE.

Table 2
Effect of treatment of CTLs generated against RL61 with antibodies to Thy-1, asialo GM1, Lyt-1, and Lyt-2 antigens and complement

<table>
<thead>
<tr>
<th>Treatment of effector cells</th>
<th>% lysis of RL61 target cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50:1⁺⁺</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>26.2 ± 0.4⁺⁺</td>
</tr>
<tr>
<td>Complement only</td>
<td>25.1 ± 1.7⁻⁻</td>
</tr>
<tr>
<td>Anti-Thy-1.2 + complement</td>
<td>2.8 ± 1.4⁻⁻</td>
</tr>
<tr>
<td>Anti-asialo GM1 + complement</td>
<td>19.3 ± 1.4⁺⁺</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>52.8 ± 0.7⁻⁻</td>
</tr>
<tr>
<td>Complement only</td>
<td>51.2 ± 0.3⁺⁺</td>
</tr>
<tr>
<td>Anti-Lyt-1.2 + complement</td>
<td>14.8 ± 0.9⁻⁻</td>
</tr>
<tr>
<td>Anti-Lyt-2.2 + complement</td>
<td>6.0 ± 0.7⁻⁻</td>
</tr>
</tbody>
</table>

⁺⁺ Effect:target cell ratio.
⁻⁻ Mean ± SE of triplicate samples.

results agree with several reports (12–14) that demonstrate the requirement of the Lyt-2⁺ T-cells to reject syngeneic tumor in mice.

The surface phenotype of the effector cells detected in our experimental system is consistent with the evidence published by other investigators about CTLs (15–17). Although the effector cells detected by the Winn assay were T-cells expressing the phenotype of CTLs these effector cells were not able to kill the RL61 cells in vitro (Tables 1 and 3). The reasons for this could be explained in two ways: (a) the cytotoxic activity of these effector cells may not be detected by the short-term assay such as a 4-h ⁵¹Cr release assay and the long-term assay may be required and (b) there is the possibility that the transition of pre-CTLs into mature CTLs may be inhibited in spleens of the tumor-bearing mice. The effector cells of the tumor-bearing mice were sensitive to irradiation of 1000 rads (Chart 3) whereas the CTLs induced by the secondary MLTC were highly resistant to irradiation (data not shown). These results indicate that proliferation and DNA synthesis of the effector cells in the tumor-bearing mice are required for acquisition of cytotoxic activity. Next as shown in Chart 3A treatment of spleen cells with anti-asialo GM1 antiserum and complement abolished their antitumor activity. However, the cytotoxic activity of the CTLs in vitro generated against RL61 was not eliminated by treatment with anti-asialo GM1 serum and complement (Table 2). Asialo GM1 has been believed to be a cell-surface marker for NK cells (18, 19). However, Beck et al. (20) reported that the precursor cells of CTLs also displayed asialo GM1 on their cell surfaces. Taken together the effector T-cells detected by the Winn assay in the present work seem to be the immature CTLs. Recently Suzuki et al. (21) reported that asialo GM1⁺ (NK) cells function as accessory cells for the induction of alloimmune CTLs in vitro and in vivo. They used anti-
asialo GM1-treated mice or spleen cells from anti-asialo GM1-treated mice as responding mice or cells, respectively. In our experiments normal mice were used as the recipients of the Winn assay. The recipient mice contain the normal number of asialo GM1+ cells. Therefore it seems possible that asialo GM1+ cells in spleen cells of the tumor-bearing mice function as the direct effector cells rather than accessory cells in our experimental system.

Recently reports that the subclass of T-cells expressing the Lyt-1+2~ phenotype mediates graft or tumor rejection have increased (4–7). Lyt-1+2~ cells are known to be responsible for triggering delayed-type hypersensitivity reactions in mice (22). Loveland et al. (6), Nelson et al. (7), and Bhan et al. (4) speculate that the mechanism by which Lyt-1+2~ cells reject graft or tumor may be through the induction of delayed-type hypersensitivity reaction. However, Lyt-1+2~ cells also have the helper or amplifier functions on CTL responses. Greenberg et al. (5) suggest that the Lyt-1+2~ cells may operate in vivo as amplifier cells rather than by direct antitumor effects. Fujitake et al. (23) reported that the helper T-cells augmented the generation of CTLs in vitro and enhanced the tumor regression in vivo. Love-land et al. (6) used adult thymectomized, lethally irradiated mice reconstituted with syngeneic T-depleted bone marrow and adoptively transferred sensitized lymphocytes and subsequently

grafted skin. Using this particular system they showed that skin rejection was dependent on the Lyt-1+2~ T-cells but independent of the presence of the Lyt-1−2~ cells. However, the recent work of LeFrancosis and Bevan (25) indicates that adult thymecto-
mized, lethally irradiated mice reconstituted with syngeneic T-depleted bone marrow contain the precursors of CTLs and that the transferred Lyt-1-2 cells function as the helper or amplifier cells of CTL precursors. There remain many questions as to whether the Lyt-1-2 T-cells are the direct effector cells against tumor growth in T-cell-deficient recipients until an adequate number of cytotoxic T-cells were generated in the draining lymph nodes of the recipients. Leclerc and Cantor (26) demonstrated that there was a correlation between the in vitro CTL activity of the Lyt-1-2 cells and their protective effect in vivo against MBL-2 lymphoma. Tyler et al. (29) proved that the cloned Lyt-1-2 CTLs were capable of destroying allogeneic tissue in vivo when they were inoculated i.d. Furthermore Evans (30) reported that large numbers of Lyt-2 lymphocytes infiltrated at the site of tumor regression. These reports present strong evidence that CTLs or CTL-lineage cells mediate graft or tumor rejection.

In conclusion we speculate that in the spleen cells of the tumor-bearing mice the pathway of differentiation and proliferation into mature CTLs may be inhibited and that these immature CTLs may differentiate and proliferate into mature CTLs which kill the tumor cells in the tumor site, i.e., under the conditions of the Winn assay.

ACKNOWLEDGMENTS

We are grateful to Dr. K. Okumura (Tokyo University School of Medicine) for providing rabbit anti-asialo GM1 serum.

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